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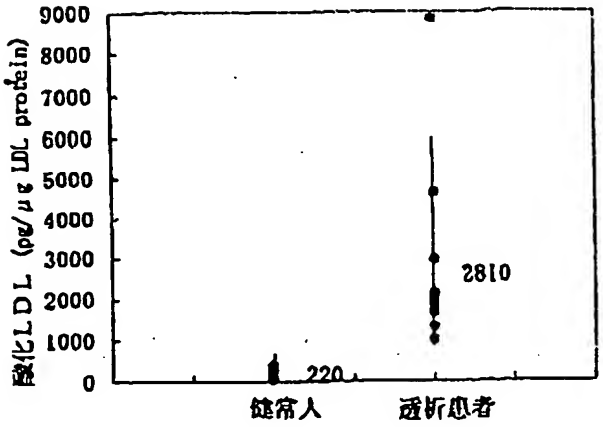
(54) [Title of the Invention] **A Method for Determination of Human Oxidized Lipoproteins**

(57) Abstract

[Objective] To provide a method for the determination of human oxidized lipoproteins whereby oxidized lipoproteins such as oxidized LDL in circulating blood are detected with high sensitivity and quantitatively by a comparatively simple method.

[Structure] It is a method for the determination of human oxidized lipoproteins whereby oxidized lipoproteins in plasma are determined using antibodies that recognize antigens that are produced by oxidation of phospholipids. When antibodies that recognize antigens that are produced by oxidation of phosphatidylcholine in the presence of peptides or antibodies that are obtained by sensitizing suitable animals by means of atherosclerotic lesions are used, particularly good results are obtained. Further, antibodies that are produced from the hybridoma cell line FOH1a/DLH3 (Registration No. FERM-14153) are particularly desirable.

*Translator's Note: Transliterated phonetically from the Japanese. As such, the spelling may differ from other transliterations.



[vertical axis]: Oxidized LDL (pg/μg LDL protein)
[horizontal axis]: Healthy persons; Dialysis patients

[Claims]

[Claim 1] A method for the determination of human oxidized lipoproteins in which oxidized lipoproteins in plasma are determined using antibodies that recognize antigens that are produced by oxidation of phospholipids.

[Claim 2] A method for the determination of human oxidized lipoproteins as described in Claim 1 in which the antibodies recognize antigens that are produced by oxidized phosphatidylcholine in the presence of peptides.

[Claim 3] A method for the determination of human oxidized lipoproteins as described in Claim 1 in which the antibodies that recognize antigens that are produced by oxidation of phospholipids are obtained by immunizing suitable mammals and/or antibody-producing carried lymphocytes of mammals with an homogenate of atherosclerotic lesions, the antibody producing lymphocytes of said animals and myeloma cells are fused, the anti-human atheroma antibody-producing fused cell group that is formed is isolated and fused cells that are selected from said cell group are produced as cells that react specifically with the oxidized human lipoproteins.

[Claim 4] A method for the determination of human oxidized lipoproteins as described in Claim 1 in which the antibodies are produced by the hybridoma cell line FOH1a/DLH3 (Registration No. FERM P-14153).

[Claim 5] A method for the determination of human oxidized lipoproteins as described in Claim 1 characterized in that plasma and/or lipoprotein separated from it is diluted to a suitable concentration, after which it is brought into contact with antibodies that recognize the antigen that is produced by oxidation of phospholipids and the oxidized lipoprotein that is connected with said antibodies is further brought into contact with the antibodies that recognize said oxidized lipoprotein.

[Claim 6] A method for the determination of human oxidized lipoproteins as described in Claim 5 characterized in that the antibodies that recognize the antigens that are produced by oxidation of the phospholipids are made into a solid phase on a carrier.

[Detailed Description of the Invention]

[0001]

[Field of industrial use] This invention relates to a method for the determination of human oxidized lipoproteins. In greater detail, this invention relates to a method for the determination of human oxidized lipoproteins in blood characterized in that blood components are brought into contact with antibodies that recognize oxidized phospholipids and in that the reactivity of said antibodies to the test material is determined. This invention further relates to a method for diagnosing various types of circulatory system diseases using the aforementioned method including coronary

artery diseases such as myocardial infarction and angina pectoris, cerebrovascular diseases such as cerebral infarction and cerebrovascular dementia, renal artery diseases such as nephrosis and diabetic nephrosis and peripheral artery diseases due to peripheral artery occlusion.

[0002]

[Prior art] Arteriosclerosis occurs frequently in the aorta, the coronary arteries, the cerebral arteries and in muscular arteries such as the carotid artery and is a disease which is the principal cause of angina pectoris, myocardial infarction and cerebral infarction. It has been proposed that its causes are elevation of serum cholesterol platelet coagulation and damage to the endothelium. However, at present, these causative factors have not been entirely explained.

[0003] Since a relationship between oxidized lipoprotein, which is a modified lipoprotein, and progress of atherosclerotic lesions was indicated by Steinberg, the problem of oxidized lipoproteins in the development of arteriosclerosis has come into the spotlight (for example, Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witzum, J.L., (1989), N. Engl. J. Med. 320: 915).

[0004] The existence has been ascertained of acceptors for lipoproteins that have undergone oxidation such as scavenger receptors. The hypothesis that foam cells are formed and that initiation of atheroma formation begins as a result of uptake of oxidized LDL into cells through the mediation of these receptors and the hypothesis that adhesion and agglutination of platelets, agglutination of leukocytes and infiltration of plasma components into the blood vessels occurs as a result of the oxidized LDL damaging the endothelial cells and that these changes serve as a trigger to bring about migration and proliferation of smooth muscle cells have been proposed.

[0005] In studies to determine whether oxidized LDL definitely accumulates in lesions Haberland, in 1968,indicated the presence of antibodies to LDL modified by malondialdehyde and that arteriosclerotic lesions are stained by anti-MDA-LDL antibodies (Herberland, M.E., Fong, D., and Cheng L., (1988) Science 241: 215). In 1989, Yla-Herttuala et al. reported that they had searched for apoB extracted from lesions by the immunoblotting method using anti-MDA-apoB antibody and that LDL that had undergone oxidative modification was definitely extracted from the lesions (Yla-Herttuala, S., Parinski, W., Rosenfield, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., and Steinberg, D., (1989), J. Clin. Invest. 84: 1086). However, the antibodies that were used in these cases were obtained using as antigens LDL that had been artificially modified using malondialdehyde and were not only oxidation products of LDL but also had the property of cross-reaction with other oxidized proteins such as, for example, oxidized albumin.

[0006] However, it has been indicated that monoclonal antibodies of high specificity can be obtained when hybridomas are prepared using homogenates of atherosclerotic lesions as the antigens and when hybridomas that produce antibodies that specifically recognize oxidized LDL are selected from them (Itabe, H., Takeshima, E., Iwasaki, H., Kimura, J., Yoshida, Y., Imanaka, T., Takano, T., (1994) J. Biol. Chem, 269 (21): 15274). Because the clone FOH1a/DLH3 is produced, these antibodies are named FOH1a/DLH3. It has been indicated that these same antibodies react specifically with oxidized lipoproteins, not exhibiting cross reactions with normal lipoproteins, LDL modified with malondialdehyde or with acetylated LDL and that the epitopes that are to be recognized by the antibodies are produced when phospholipds such as phosphatidylcholine, which are structural components of lipoproteins, are oxidized. It has also been indicated that these same antibodies are antibodies that specifically recognize foam cells in human atherosclerotic lesions.

[0007] In research up to the present, it has been concluded that oxidation of LDL is brought about by secondary chemical modification after deposition in vascular tissue. There is also the possibility that there are lipoproteins that have undergone oxidative modification in the circulating blood due to active oxygen that is produced at sites of inflammation. In actuality, lipids have been extracted from human blood or from its LDL fraction, the presence of phospholipid peroxides in them has been demonstrated and it has been reported

that they are elevated during such diseases as cardiac ischemia, diabetes and hepatitis (Miyazawa, T., (1989), Free Radical Biology 7: 209, Hodis, H.N., Kramsch, D.M., Avogaro, P., Bittolo-Bon, G., Cazzolato, G., Hwang, J., Peterson, H., and Sevanian, A., (1994) J. Lipids Res., 35: 669). However, the method of determination is complex, large numbers of clinical specimens have to be determined and it is not suited to clarifying the clinical diagnostic significance of oxidized lipoproteins in the blood, for which reason at present the relationship between oxidized lipoproteins in the blood and disease has not yet been clarified. If oxidized LDL is deeply related to progress of atherosclerotic lesions, it is clear that detection of oxidized lipoproteins such as oxidized LDL in circulating blood in high sensitivity and quantitatively must be established in early diagnosis of advances of pathological states. The development of such methods is strongly desired.

[0008]

[Problems the invention is intended to solve] Consequently, this invention has the objective of providing a novel method for the determination of human oxidized lipoproteins. This invention has the further objective of providing a method for the determination of human oxidized lipoproteins whereby oxidized lipoproteins such as oxidized LDL in the circulating blood are detected with high sensitivity and quantitatively by a comparatively simple procedure. This invention also has the objective of providing a method for diagnosing various types of circulatory system diseases using the aforementioned method including coronary artery diseases such as myocardial infarction and angina pectoris, cerebrovascular diseases such as cerebral infarction and cerebrovascular dementia, renal artery diseases such as nephrosis and diabetic nephrosis and peripheral artery diseases due to peripheral artery occlusion.

[0009]

[Means for solving the problem] The aforementioned objectives are achieved by a method for the determination of human oxidized lipoproteins whereby oxidized lipoproteins in plasma are determined using antibodies that recognize antigens that are produced by oxidation of phospholipids.

[0010] This invention is also a method for the determination of human oxidized lipoproteins in which the antibodies are substances that recognize the antigens that are produced by oxidation of phosphatidylcholine in the presence of peptides. This invention is, further, a method for the determination of human oxidized lipoproteins in which the antibodies that recognize antigens that are produced by oxidation of phospholipids are obtained by immunizing suitable mammals and/or antibody-producing carried lymphocytes of mammals with an homogenate of atherosclerotic lesions, the antibody producing lymphocytes of said animals and myeloma cells are fused, the anti-human atheroma antibody-producing fused cell group that is formed is isolated and fused cells that are selected from said cell group are produced as cells that react specifically with the oxidized human lipoproteins. This invention is also a method for the determination of human oxidized lipoproteins in which the antibodies are produced by the hybridoma cell line FOH1a/DLH3 (Registration No. FERM P-14153). This invention is also a method for the determination of human oxidized lipoproteins characterized in that plasma and/or lipoprotein separated from it is diluted to a suitable concentration, after which it is brought into contact with antibodies that recognize the antigen that is produced by oxidation of phospholipids and the oxidized lipopotein that is connected with said antibodies is further brought into contact with the antibodies that recognize said oxidized lipoprotein. This invention is, further, a method for the determination of human oxidized lipoproteins characterized in that the antibodies that recognize the antigens that are produced by oxidation of the phospholipids are made into solid phase on a carrier.

[0011]

[Action] In order to solve the problems described above, a method is necessary in which epitopes are clear and which is of high specificity for oxidized LDL and in which individual quantitative determinations of various types of lipoproteins are made. The inventors ascertained that the antibody FOH1a/DLH3, which is obtained using atherosclerotic lesions as the antigen, recognizes epitopes that are produced by oxidation of phospholipids (Japanese

Patent Application Announcement No. 6-51,209 [1994], Itable, H., Takeshina, E., Iwasaki, H., Kimura, J., Yoshida, Y., Imanaka, T., Takano, T., (1994) J. Biol. Chem. 269 (21): 15274)).

[0012] The inventors conducted intensive research on such antibodies. As a result, they arrived at this invention by discovering that such antibodies are the best antibodies for providing the required method of determination as described above.

[0013] Specifically, detection of oxidized lipoproteins with high sensitivity and quantitatively is obtained by using antibodies that recognize antigens that are produced by oxidation of phospholipids. In particular, it is clear from these effects that there are cases in which these antibodies are actually obtained by sensitizing suitable animals with atherosclerotic lesions and there are cases in which the epitope that recognizes the antigen originates in a structure that is produced by oxidation of phosphatidylcholine in the presence of peptides.

[0014] The reason that such antibodies are excellent for solving the aforementioned problems is that antibodies having the properties described below are obtained. The antigens of such antibodies are manifested by production of lipoproteins that actually occur in human tissues. Moreover, there is also an extremely high possibility that they are produced by the oxidation of lipoproteins which are found in both peptides and phospholipids in plasma proteins. The antibodies produced by the hybridoma FOH1a/DLH3 are endowed precisely with these properties.

[0015] When determinations are made using these antibodies, the objective is achieved by bringing plasma and/or serum into direct contact with the antibodies. When the sandwich ELISA method, to be described subsequently, is used, the sample may be fractionated in advance up to the lipoprotein fraction by a suitable method (for example, ultracentrifugation) in order to prevent nonspecific adsorption attributable to this determination method.

[0016] Second, because the epitope that recognizes these antibodies is not dependent on the apoprotein, a method can be provided for the individual evaluation of oxides of different lipoproteins in the blood. For this purpose, two types of antibodies are necessary, oxidized phospholipid specific antibodies and said lipoprotein specific antibodies. At this time, the so-called sandwich ELISA method in which either of these antibodies is made into solid phase with a plate-shaped or spherical carrier such as a plastic plate or glass beads is convenient.

[0017] At this time, there is no particular limitation on which of the antibodies is made into solid phase. However, in the case in which the antibody titer of the antibodies that recognize the oxidized phospholipids is high, making these antibodies into solid phase is advantageous from the standpoints that the antigens can be concentrated and that high sensitivity can be achieved. In the examples, an example of the ELISA method is presented in which the antibodies that are produced by the hybridoma FOH1a/DLH3 are made into solid phase and prepared. However, it goes without saying that this invention is not limited to this example.

[0018] Third, by using such antibodies, as shown in the examples, for example, by using oxidized LDL prepared artificially as the standard substance, the value can be evaluated, for example, quantitatively in the form of the ng quantity of oxidized LDL per 1 μg of LDL protein.

[0019] This invention is the first that is provided in a form in which the degree of oxidation of each individual lipoprotein can be differentiated with high specificity and high sensitivity and with which quantifiable determinations of oxidized LDL in the blood can be made simply, and, therefore, of large numbers of clinical samples, by the ELISA method.

[0020] We shall now describe this invention in greater detail on the basis of its mode of execution.

[0021] The method of determination of this invention is characterized in that the blood components to be tested and the antibodies that recognize the

antigens that are produced by oxidation of phospholipids as described above are brought into contact and in that the quantity of antigen that reacts specifically with said antibodies is determined quantitatively, by which means the oxidized lipoproteins in the blood are ascertained. The determinations can be performed on the basis of known methods such as the RIA method, the ELISA method, the immunoblotting method and the immunoprecipitation method.

[0022] Further, because the antibodies of this invention as described above that recognize the antigens that are produced by oxidation of phospholipids as described above are not dependent on the apoproteins of the recognizing epitopes, oxides of different lipoproteins in the blood can be evaluated individually. Antibodies that recognize one or two or more of chylomicron, VLDL, LDL, HDL2, HDL3 or Lp(a) can be used as the specific antibodies for lipoproteins. Of these, evaluation of oxidized LDL is particularly important because of the relationship between oxidized LDL and atherosclerosis. In addition, it is also important to evaluate whether or not there are oxidative changes of Lp(a), to which attention has been drawn most recently as an independent risk factor of arteriosclerosis (for example, Scanu, A.M., Lawn, R.M., and Berg K. (1991) Lipoporotein (a) and athergenesis, Ann. Int. Med. 115: 209-218). The antibodies to these lipoproteins can be commercially sold products or they can be acquired or prepared easily by known methods. Thus, when oxides of different lipoproteins in the blood are to be evaluated individually, it is desirable to make either the oxidized phospholipid specific and lipoprotein specific antibodies as described above, and, preferably, antibodies of high antibody titers into solid phase in plate form or spherical form and it is particularly desirable to use the sandwich ELISA method. For example, carriers that can be used in making the solid phase can include apparatuses made of plastic or glass that are commonly used in these fields such as multiple hole plates and glass beads.

[0023] The blood components that are the test materials for which determinations are to be made are plasma or serum components that are obtained by collecting blood from the test sample, and, preferably, by drawing blood to which an anticoagulant such as heparin has been added and separating the components from the blood samples that were obtained by a standard method such as centrifugation. Further, in order to inhibit nonspecific adsorption attributable to the determination method and to make determinations of higher accuracy, the plasma components may be further separated by ultracentrifugation and obtained as lipoprotein fractions.

[0024] Further, in determination, these plasma and/or lipoprotein fractions are diluted to the optimum concentrations. Because the concentration is controlled by the determination conditions, it cannot be specified in general terms. For example, they are diluted to 0 - 500 μg/ml, and, more preferably, to 0 - 100 μg/ml. There are no particular limitations on the dilution medium. For example, physiological saline solution and phosphate buffer solution (PBS) containing EDTA can be used.

[0025] There are no particular limitations on the contact between the plasma and/or lipoprotein fractions that have been set to the optimum concentration and the oxidized phospholipid specific antibodies described above as long as the specific reaction between the oxidized phospholipid and the oxidized phospholipid specific antigen contained these blood components. For example, the reaction may be carried out with the materials being allowed to stand at 4 to 30°C, and, preferably, at below 25°C, for 1 to 24 hours, and, preferably, for 1 to 2 hours. The concentration of the oxidized phospholipid specific antigen at this time may be an amount that is sufficiently supersaturated relative to the amount of phospholipid that is thought to be present in the blood test material. Further, the antibody titer of the antibodies is controlled by the type of determination method. For example, in the case in which the quantity of oxidized LDL present per 1 μg of plasma is estimated to be on the order of 0 to 1 ng and when DLH3 antibodies as described subsequently are used as the oxidized phospholipid specific antibodies, it should be 0.2 to 1.0 μg, and, preferably, about 0.5 μg, per 1 μg of plasma.

[0026] We shall now present a detailed description of the antibodies that recognize the antigens that are produced by oxidation of the phospholipids that are used in the method of determination of this invention.

[0027] There are no particular limitations on the method whereby these antibodies are obtained. However, preferably, they are produced from a hybridoma cell line that is obtained, as described below, by a method in which suitable animals are sensitized by athersclerotic lesions or by a procedure as described below based on general cell fusion methods.

[0028] There are no particular limitations on the species of animals that can be used in preparation of the hybridoma and mice, rats and hamsters, which are used conventionally, may be used. However, Balb/c mice are desirable because they are easily acquired and are easy to handle and spleen cells of these animals are used for the most part. In addition, human lymph node cells and peripheral lymphocytes can also be used.

[0029] The antigen for immunization of these animals is prepared from atherosclerotic lesions. For example, blood vessels that have undergone pathological changes are acquired on autopsy immediately after death from patients with arteriosclerosis or at by-pass surgery, the blood vessels sites containing the atherosclerotic lesions are excised from the blood vessels exhibiting pathological changes and the tunica adventitia is stripped off and removed in buffer solution, after which the tunica intima with the lesion and the tunica media are cooled using an homogenizer, or, preferably, are homogenized under ice water cooling and are allowed to stand. Following that, the supernatant that is obtained is used as the antigen solution. As required, centrifugation can be performed after they have been allowed to stand, buffer solution is added to the pellets and the same procedure is performed. The supernatant that is obtained is combined with the previous supernatant and can also be used as the antigen solution. The antigen solution that has been prepared in this way should be allowed to stand in an inert gas such as, for example, argon and should be frozen and stored until immediately before use.

[0030] Next, the antigen comprised of the homogenate of atherosclerotic lesion that has been prepared in this way is set to a specified protein (antigen) concentration and is used to immunize the animals described above. At this time, as required, adjuvants such as Freund's complete adjuvant or Freund's incomplete adjuvant may be added.

[0031] The quantity administered is controlled by species of animal. In the case of mice, on the initial immunization, it should be on the order of 2.0 to 60 μg (protein) / animal, and, preferably, 40 μg (protein) / animal.

[0032] After the initial immunization, for example, at intervals of 2 weeks and 4 weeks, it is desirable to perform an additional immunization with a quantity of protein of the same or smaller quantity as in the initial immunization.

[0033] Blood is drawn from the immunized animals 2 to 3 days after the final immunization, confirmation of elevation of serum antibody titer is performed by a detection method such as the ELISA (enzyme-linked immunoabsorbent assay) method or immunoblotting method and immunized animals in which antibody titer elevations are found are screened.

[0034] Spleen cells from the immunized animals that have been screened or antibody-producing cells from the lymph nodes are collected. They are then washed and suspended in a maintenance culture medium such as RPMI culture medium or DMEM culture medium that has been heated to approximately 37°C and cell counts are measured.

[0035] On the other hand, tumor cells of HGPRT (hypoxanthine-guanine phosphoribosyl transferase) deficient strains are grown in a growth medium such as RPMI medium to which fetal cow serum (FCS) is added or DMEM medium to which FCS is added and are cultured to the logarithmic growth phase. Known tumor cells such as, for example, P3/X63-Ag8 (X63) (the entries in parentheses being the abbreviated names; the same hereafter), P3/NSI-1-Ag4-1(NS-1), P3/X63-Ag8.U1 (P3/U1), Sp2/0-Ag14 (Sp2/O), F0, 210.RCY3.Ag 1.2.3. (Y3), U-266AR1 (SKO-007), LICR-LON-Hmy2 (Hmy2) and 8226AR/NIP4-1 (NP41) can be used, depending on the species of animal, as the tumor cells of HGPRT deficient strains. Tumor cells in the logarithmic growth phase are regulated so that the cell count of tumor cells relative to the cell count of the aforementioned antibody producing cells is 1 : 1 - 1 : 10. They are washed in a maintenance

medium such as RPMI medium or DE<E medium that has been heated to approximately 37°C and the FCS component, which impedes cell fusion, is removed.

[0036] The antibody producing cells and tumor cells the cell counts of which have been adjusted are mixed in a container such as, for example, a glass tube and are centrifuged, with pellets being obtained. The supernatant is removed as much as possible. The subsequent procedures, as well as this procedure, should be performed at temperature conditions of 20 to 37°C, and, preferably, of approximately 37°C.

[0037] Next a cell agglutinating medium that has been heated to 0 - 37°C, and, preferably, to approximately 37°C, is added slowly to the pellets that are obtained as the pellets are being disentangled. The substances that can be used as the cell agglutinating medium include compounds such as polyethylene glycol (PEG), lysolecithin and glycerol oleic acid esters and inactivated Sendai virus (HVJ), measles virus or paramyxoviruses such as Newcastle disease virus. Of these, PEG is preferable. When PEG is used, for example, with RPMI medium or DMEM medium, it should be diluted to a concentration on the order of 45 to 50 wt % in the case of PEG 4000, although this depends on the average molecular weight.

[0038] After addition of the cell agglutinating medium, stirring is continued for another 1 to 2 minutes, after which a maintenance medium such as an RPMI medium is added slowly on 2 to 3 separate occasions.

[0039] Following that, centrifugation is performed under weak conditions, for example, 800 to 1200 × g for 3 to 5 minutes, in order to remove the cell agglutinating medium such as PEG and the supernatant is removed.

[0040] Next, as the pellets that have been obtained are being disentangled, a selective medium such as HAT medium to which FCS has been added is added slowly so that the spleen cell concentration becomes 1×10^6 to 1×10^7 cells/ml, the materials are injected into each well of a multiple hole plate such as a 96 hole plate and culture is performed under conditions of a temperature of approximately 37°C, a CO_2 concentration of approximately 7% and a humidity of 100%. During the culture period, depending on the state of the cells, the solution is replaced at intervals of about 2 to 3 days. The culture conditions are not limited to those described above. In addition, for example, a growth medium such as RPMI culture medium to which FCS has been added can be initially added to the pellets, and, after culture has begun, selective medium can be added to each well. Cells that have not fused begin to die rapidly from the third day and die completely by about day 7. On the other hand, cells that have succeeded in fusing, i.e., hybridomas, begin to form colonies from this point on. Screening as described next is begun from the wells in which formation of hybridoma colonies has been found. As required, they are subcultured in large plates such as 24-hole plates.

[0041] Screening can be performed by the RIA method, the ELISA method or the immunoblotting method. Of these, the ELISA method is preferable. Oxidized LDL obtained by a reaction of 3 hours or longer with $CuSO_4$ is used as the antigen. As required, undenatured LDL may be used in combination. Antigen that is produced by oxidation of phosphatidylcholine is particularly desirable and it is desirable that it be recognized in the presence of peptides. On the basis of the individual assay methods, the culture supernatant that has been collected from wells in which hybridoma colony formation has been found is screened and cell strains are selected for which there are positive findings in reactions with oxidized LDL (and negative findings in reactions with undenatured LDL).

[0042] Cloning is performed directly from wells in which there are positive findings on screening. Cloning can be performed using the limiting dilution method and the single cell manipulation method. The limiting dilution method is preferable because it is technologically simpler.

[0043] When the cloned cells are again grown, they are screened in the same way as described above and cloning is repeated. A high production cell strain that does not react with the unmodified LDL and that reacts only with the oxidized LDL is identified.

[0044] There are no particular limitations on the method of storage of the hybridoma that is obtained. For example, a method may be used in which as large a number as possible of cells, for example, on the order of 1×10^7 to 2×10^7 cells, is suspended in 1 to 2 ml of 90% FCS and 10% dimethyl sulfoxide (DMSO) in a vial for freeze storage and the suspension is frozen and stored in liquid nitrogen.

[0045] The fact that cell strains that do not react with unmodified LDL and that react only with oxidized LDL can be obtained by the cell fusion procedure described above using Balb/c mice is disclosed in the paper mentioned above (Itabe, H., Takeshima, E., Iwasaki, H., Kimura, J., Yoshida, Y., Imanaka, T., Takano, T., (1994) J. Biol. Chem, 269 (21): 15274). The antibody FOH1a/DLH3, which is described in the same paper, is a particularly desirable monoclonal antibody in that it does not react with malondialdehyde modified LDL (MDA-LDL) and acetylated LDL (AcLDL). The mouse-mouse hybridoma cell line FOH1a/DLH3 that produces this antibody is registered in the Life Engineering Industrial Technology Institute of the Agency of Industrial Science and Technology and has been given the Registration Number FERM P-14153.

[0046] The monoclonal antibody FOH1a/DLH3 reacts with artificially oxidized LDL using LDL and copper ions. However, it does not react with unmodified LDL and does not react with LDL when it has been modified by other methods (for example, morondialdehyde addition, acetylation, etc.). It also does not react when other serum proteins, for example, albumin and globulin, that have been oxidized. However, it reacts when high density lipoprotein, (HDL), which is a lipoprotein different from LDL, is oxidized.

[0047] However, the method for obtaining antibodies that recognize the antigen that is produced by oxidation of phospholipids of this invention is not limited to methods of sensitizing suitable animals with athersclerotic lesions as described above. Other methods that can be considered can include, for example, a method in which oxidized LDL is used as the immunogen and a method in which the immunogen is obtained by oxidizing a phospholipid in the presence of an apoprotein or a portion of its structural peptides.

[0048] The following can be considered as the conditions of production of artificial oxidized lipoprotein that is required in the method of determination of this invention or in obtaining the hybridoma that produces the antibodies used in the method of determination. Specifically, the lipoprotein fraction is obtained from normal human serum, for example, by the centrifugation precipitation method, and this fraction, as required, is subjected to purification treatment by dialysis or desalting, after which $CuSO_4$ is added to the lipoprotein reaction in proportions of a protein concentration of 0.1 to 1 mg/ml, and, preferably, 0.2 mg/ml, and a $CuSO_4$ concentration of 5 to 25 μm, and, preferably, 5 μm, and a reaction is carried out for 3 to 24 hours at approximately 37°C.

[0049]

[Examples] We shall now present examples to describe the sandwich ELISA analysis method for oxidized lipoproteins of this invention in more specific terms.

[[0051] Example 1

Sandwich ELISA analysis method for oxidized LDL

(1) Preparation of LDL fraction in human serum

EDTA was added to human plasma that was obtained by collection with heparin to give a final concentration of 0.25 mM, amounts of 0.75 ml each were collected into test tubes (1 to 4 ml capacity) for ultracentrifugation, 250 μl of 0.15M NaCl containing 0.3 mM EDTA was made into a multiple layer and centrifugation was performed at 185,000 × g for 2.5 hours at 10°C. 150 μl of the top layer was discarded and 750 μl of the bottom layer was collected and 150 μl of KBr solution (50 w/v %) was added to give a specific gravity of 1.063. Plasma, the specific gravity of which had been adjusted, was transferred to the bottom of test tubes (1 to 4 ml capacity) for ultracentrifugation and the materials were centrifuged at 244,000 × g for 16 hours at 10°C. The orange band (approximately 100 to 150 μl) in the top layer was recovered with great care

and it was dialyzed at 40°C for 6 hours (with amounts of 3 liters being replaced two times at 1 hour intervals) against PBS containing 0.25 mM EDTA. Quantitative determinations were made of the protein and cholesterol in the LDL test material that was obtained

[0051] (2) Sandwich ELISA analysis

DLH3 antibodies and IgM antibodies of unimmunized mice (0.6 μg each/well) diluted with PBS were added to plates and the materials were allowed to stand for 2 hours at room temperature. Next, 200 μl of 1% BSA-TBS solution (pH 7.4) was added, the mixture was allowed to stand for two hours at room temperature and blocking occurred. The blocking solution was discarded, oxidized LDL standard product in unaltered form and human LDL fraction were injected (the oxidized LDL standard product consisting of 0.1 to 20 ng of LDL protein/well; the human LDL fraction consisting of 2 μg of LDL protein/well) and the materials were allowed to stand at 4°C for 18 hours. The materials were washed three times with 0.05% Tween 20-TBS (pH 7.4), after which 100 μl of sheep anti-human apo B antibody (Bindind Site Company) diluted 5000 times was added and the materials were allowed to stand for 2 hours at room temperature. They were then washed three times with 0.05% Tween 20-TBS pH 7.4), after which 100 μl of alkaline phosphatase-labeled donkey anti-sheep IgG antibody (manufactured by the Chemicon Company) diluted 2000 times with 2% skimmed milk was added. The materials were then allowed to stand for two hours at room temperature, after which they were washed three times with 0.05% Tween 20-TBS (pH 7.4). 100 μl of 0.1% p-nitrophenylphosphoric acid (pH 8.8) was added, with coloration being effected, and absorbance at 405 nm was determined after 10 to 60 minutes.

(3) Results of analysis using human plasma (from healthy individuals and kidney dialysis patients)

Figure 1 and Figure 2, respectively, show the typical calibration curve using oxidized LDL as the standard product and the results of analysis of clinical samples.

[0052] Example 2

Sandwich ELISA analysis of oxidized Lp(a)

(1) Preparation of peroxidase-labeled anti-Lp(a) antibodies

EDTA was added to human plasma obtained by collecting blood with heparin to give a final concentration of 0.25 mM, 250 μl of 0.15M NaCl containing 0.3 mM EDTA was made into a double layer and centrifugation was performed at 105,000 × g for 20 hours at 8°C. The top layer was discarded, KBr that had been pulverized in advance with a mortar was added to the bottom layer, the mixture was dissolved at 4°C so that foaming did not occur and it was prepared to a specific gravity of 1.125. Centrifugation was then performed at 105,000 × g for 20 hours at 8°C. The orange band of the top layer was recovered with great care and gel filtration was performed using Biogel A-5m with 1M NaCl, 2mM EDTA and 10 mM phosphate buffer solution as the developing solvent. Each fraction that was obtained was determined using an Lp(a) determination kit manufactured by the Thermo [phonetic] Company, Ltd., and the Lp(a) fraction was recovered. This fraction was applied to lysine Sepharose 4B manufactured by Pharmacia, the adsorbed fraction was eluted with buffer solution containing 0.2M ε-aminocaproic acid and was dialyzed against PBS containing 0.25 mM EDTA, with the Lp(a) fraction being obtained. A rabbit was immunized with 0.5 mg of the Lp(a) that was obtained and antiserum was prepared. The anti-Lp(a) serum that was obtained was purified to IgG using a protein G column manufactured by Pharmacia and was passed through an LDL column that had been prepared separately, with the anti-Lp(a) antibodies being removed and anti-Lp(a) antibodies being obtained. The purified anti-Lp(a) antibodies were labeled with peroxidase by the maleimide method.

[0053] Preparation of lipoprotein fractions in human plasma

EDTA was added to human plasma obtained from blood collected with heparin to give a final concentration of 0.25 mM. Amounts of 0.75 ml were collected in test tunes (1 ml capacity) for use in ultracentrifugation, 250 μl of 0.15M NaCl containing 0.3 mM EDTA was made into a double layer and centrifugation

was performed at 185,000 × g for 2.5 hours at 10°C. 150 μl of the top layer was discarded, 750 μl of the bottom layer was collected, KBr (70.0 mg) that had been pulverized in advance with a mortar was added and was dissolved at 4°C so that foaming did not occur. Plasma, the specific gravity of which had been adjusted (d = 1.12), was transferred to the bottom test tubes (1 ml capacity) for use in ultracentrifugation and centrifugation was performed at 244,000 × g for 16 hours at 10°C. The orange band of the top layer (approximately 100 to 150 μl) was recovered with great care and was dialized at 4°C for 6 hours (amounts of 3 liters being replaced two times at two-hour intervals) against PBS containing 0.25 mM EDTA. Quantitative determinations were made of the protein and cholesterol in the lipoprotein fraction that was obtained.

[0054] (3) Sandwich ELISA analysis

Partially purified FOH1a/DLH3 antibodies and unimmunized rat IgM antibodies that had been diluted with PBS were added to a plate (0.6 μg of each/well) and the materials were allowed to stand for two hours at room temperature. Next, 200 μl of 1% BSA-TBS solution (pH 7.4) was added and the mixture was allowed to stand for 2 hours at room temperature, with blocking occurring. The blocking solution was discarded, oxidized Lp(a) standard product in unaltered form and the human lipoprotein fraction were injected (oxidized Lp(a) standard product in amounts of 0.1 to 10 ng/well; the human lipoprotein fraction as a solution diluted 20 times with PBS) and the materials were allowed to stand for two hours at room temperature. They were then washed three times with 0.05% Tween 20-TBS (pH 7.4), after which 100 μl of peroxidase-labeled anti-Lp(a) polyclonal antibodies diluted 2000 times with 2% skim milk solution were added and the materials were allowed to stand for one hour at room temperature. They were then washed three times with 0.05% Tween 20-TBS (pH 7.4), after which 100 μl of 0.03% aqueous solution of hydrogen peroxide containing 3 mg/ml of o-phenylenediamine, with coloration being effected. After 10 to 15 minutes, the reaction was stopped with 50 μl of 2N sulfuric acid and absorbance at 492 nm was determined.

[0055] (4) Results of analysis using human plasma (patients with vascular system diseases of the kidneys)

Figure 3 and Figure 4, respectively, show the typical calibration curve using oxidized Lp(a) as the standard product and the results of analysis of clinical samples.

[0056]

[Effect of the invention] This invention, as described above, relates to a method for the detection of oxidized lipoproteins in blood characterized in that blood components and antibodies that recognize oxidized lipophosphates are brought into contact and in that the reactivity of said test antibodies to said test materials is determined, and, further, it relates to a method for diagnosing various circulatory system diseases in which atherosclerosis is the principal factor. The term circulatory system disease refers to all circulatory system diseases including coronary artery diseases such as myocardial infarction and angina pectoris, cerebral arterial diseases such as cerebral infarction and cerebrovascular dementia, renal artery diseases such as nephrosis and diabetic nephrosis and peripheral artery diseases such as peripheral artery obstruction. As shown in the examples, by means of this invention, high concentrations of oxidized LDL and oxidized Lp(a) were detected in the blood of patients treated by blood dialysis because of diabetic nephrosis and the cause-and-effect relationship between these diseases and oxidized LDL and oxidized Lp(a) was ascertained. However, the effectiveness of this invention is not limited to these aspects and is perhaps applicable to all diseases in which oxidized lipoproteins are involved. It goes without saying that the range of diseases will be expanded by future clinical studies using this invention.

[Brief Explanation of the Figures]

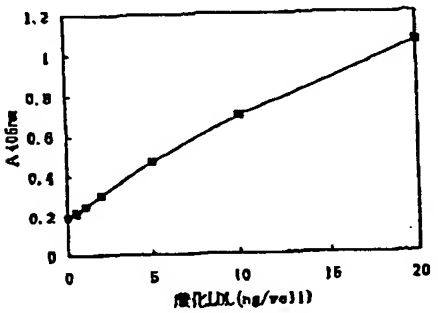
[Figure 1] This is a calibration curve obtained using the oxidized LDL obtained in Example 1 of this invention as the standard product.

[Figure 2] This is a graph showing the results of analysis of clinical samples in Example 1 of this invention.

[Figure 3] This is a calibration curve obtained using the oxidized Lp(a) obtained in Example 2 of this invention as the standard product.

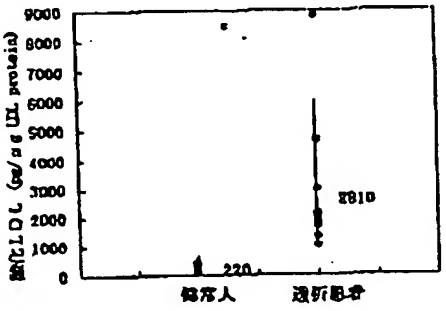
[Figure 4] This is a graph showing the results of analysis of clinical samples in Example 2 of this invention.

Figure 1



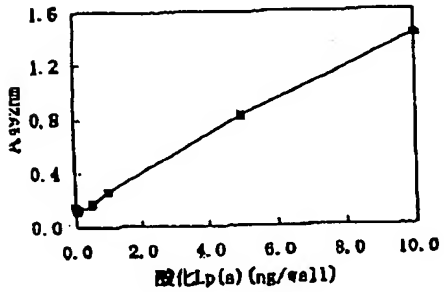
[horizontal axis]:
Oxidized LDL (ng/well)

Figure 2



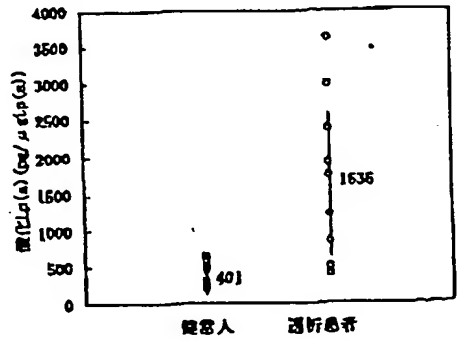
[vertical axis]: Oxidized LDL (pg/μg LDL protein)
[horizontal axis]: Normal persons; Dialysis patients

Figure 3



[horizontal axis]: Oxidized Lp(a) (ng/well)

Figure 4



[vertical axis]: Oxidized Lp(a) (pg/μg Lp(a))
[horizontal axis]: Normal persons; Dialysis patients

Continued from front page

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